Agent for gene therapy and for the prevention of metastases as well as for the gene therapy of tumours

Description

Far and away the most frequent cause of death in malign tumour diseases is organ metastasasation. Whereas the primary tumour can at least be resected surgically in early and middle stages, this is rarely possible in the event of metastasasation. Apart from a few exceptions, conventional methods of chemotherapy and radiation can, if anything, only achieve temporary improvements of the clinical picture of metastasased tumours.

Colorectal carcinomas are amongst the most frequent tumours. Hepatic metastases of a colorectal origin are the most frequent cause of death for patients with colorectal carcinomas. As they frequently portray the sole manifestation of the disease for a long time following surgical removal, they are a possible target for curative therapeutic approaches (Dreben, JA and Niederhuber, JE. (1993) Cancer of the lower gastrointestinal tract - Colon cancer. In: Niederhuber, JE ed. Current Therapy in Oncology. St. Louis, MO: Decker, 426-431). The potentially curative surgical removal of hepatic metastases is only possible for a small percentage of the patients, and temporary remission, but no extension of life, is indicated for chemotherapy. For this reason, there is an urgent need for alternative forms of therapy.

The gene-therapeutic approaches which have been in development for about 10 years have a dramatically higher degree of controllability in comparison with conventional therapeutic forms due to their complexity. They can be used in the area of cancer gene therapy, inter alia for tumour-specific targeting of the vectors or for limitation of the gene expression to tumour tissue and for specific adaptation of the transferred transgenes to points of attack of the type of tumour in question.

Apart from immunological approaches, the gene-therapeutic approaches produced up to now, as well as conventional methods before them, almost exclusively have the infiltrating tumour tissue as their primary point of attack. This causes the problem of insufficient reaching of the tumour, which presents itself with a increased intra-

1

tumoral pressure and limited supply of blood and is only insufficiently hit, even in the direct intra-tumoral injection of gene-therapeutic transfer vehicles customarily done. In cases of multiple metastasasation, it has been seen that the surrounding normal tissue can even be infected considerably better than the tumour cells with the systemic or regional administration of the vectors which is then necessary.

The invention has the objective of improving the prophylaxis and the therapy of tumour metastases and primary tumours.

The invention is achieved according to the claims. The strategy developed according to the invention avoids the problems of the difficulty in reaching tumour tissue by choosing the normal tissue, which is easy to reach, being declared the primary gene-therapeutic target. The method excels in that the normal organ tissue in equipped in situ with a potential or completed metastasasation with a defensive function, which prevents an establishment and further growth of the metastases. Likewise, the further propagation of an inoperable primary tumour can be prevented. This strategy of the impregnation of the healthy tissue decidedly differs from all the gene-therapeutic and non-gene-therapeutic approaches carried out up to now. Compared with systemic or intra-peritoneal application, e.g. of synthetic protease inhibitors (Nelson, N.J. (1998) Inhibitors of Angiogenesis enter phase III testing, J Natl. Cancer Inst., 90, 960-963), the advantage of the gene-therapeutic approach is achieving very high concentrations of active agent locally in the target organ with the benefit of lessening side-effects and of the possibility of simultaneous application of a number of inhibitors (see below). In addition, lasting gene expression after a single vector application is cheaper and lower in side-effects than repeated administration of synthetic substances for a long period of time.

The approach described below can be included in the following clinical scenarios:

1. Pre-operative or intra-operative vector application into target organs, in order to suppress the extravasion of any metastatic tumour cells released in the operation of the primary tumour.

2

2. The adjuvant expression of inhibitors in the target tissue continuing for a number of years to prevent the expansion or to kill off occult micro-metastases, above all in high-risk groups and operated mamma carcinomas with attacks of the lymphatic nodes.

3. Limitation of the growth of metastases already established or inoperable primary tumours.

Embodiment 1: Gene therapy of colorectal hepatic metastases by adenoviral transfer of metalloproteinase inhibitors (TIMPs) into the liver parenchyma

Metastases of colorectal carcinomas produce various proteases which they need for the intravasion and extravasion and for invasion in the target tissue. They include various metalloproteinases (MMPs), here in particular MMP-2 and MMP-9, which are responsible for the degradation of components of the extra-cellular matrix (ECM) (Duffy, M.J. and McCarthy, K. (1998) Matrix metalloproteinases in cancer: Prognostic markers and targets for therapy (review), Int. J. Oncol., 12, 1343-48). Of the MMPs-2 and -9, preferably collagen IV is decomposed as the main product of the basal membranes. With this knowledge, synthetic protease inhibitors were developed, which have already been used clinically and in some cases have already reached phase III clinical testing (Nelson, N.J. (1998) Inhibitors of Angiogenesis enter phase III testing, J Natl. Cancer Inst., 90, 960-963).

The basic therapeutic idea developed according to the invention comprises having inhibition agents of metalloproteinases secreted by the hepatic parenchyma in order to inhibit the extravasion of metastatic tumour cells, to suppress the further infiltration of metastases already established and to interrupt the supply of the tumour with vessels by inhibiting vessel development. To start with, the tissue inhibitor of metalloproteinase 2 (TIMP-2) was selected. This inhibitor, just like a number of related agents, physiologically ensures a limitation of the MMP activity in conversion processes. By binding to MMP-2, TIMP-2 can inhibit their activity.

As a vector for the gene transfer, first-generation adenoviruses, which are amongst the most established gene transfer systems (Brand, K. and Strauss, M (1998) Molekulare Grundlagen des Gentransfers und Anwendung für die Gentherapie. In:

3

Ruckpaul, D. und Ganten, D. (Hrsg.) Handbuch der Molekularen Medizin, Bd 2 Tumorerkrankungen, Springer, Berlin, Heidelberg, New York) are used. These vectors, which cannot replicate due to the lack of the adenoviral E1 gene, infect epithelial cells with high efficiency and can be also be generated in the large quantities necessary.

a) To detect the secretion of TIMP-2 by hepatocytes in vitro, A2 cells (hepatocyte cell line from 53 k.o mice) were infected with various multiplicities of infection (MOIs) Ad-TIMP-2. The construction of Ad-TIMP-2 has been described (Baker, A.H., Wilkinson, G.W., Hembry, R.M., Murphy, G., and Newby, A.C. (1996) Development of recombinant adenoviruses that drive high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells. Matrix Biol. 15: 383-395). The virus contains human TIMP-2 cDNA under the control of the CMV promoter. 24h or 48h later, the cell culture supernatant (CCS) was obtained. 10 µl was applied to a 10% polyacryl amide gel and transferred to a nitro-cellulose membrane after electrophoretic separation. A monoclonal antibody (T2-101, Ab-1 vonDianova, Hamburg, Germany) was used for immunological detection.
The Western Blot showed a strong TIMP-2 band in the supernatant of Ad-TIMP-2 infected cells and no bands in non-infected and control virus infected supernatants.

b) The functionality of TIMP-2 was examined by means of reverse zymography (to Baker, A.H., Wilkinson, G.W., Hembry, R.M., Murphy, G., and Newby, A.C. (1996) Development of recombinant adenoviruses that drive high level expression of the human metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and -2 genes: characterization of their infection into rabbit smooth muscle cells and human MCF-7 adenocarcinoma cells. Matrix Biol. 15: 383-395). Cells were infected as described under (a). The CCS was concentrated by means of an Amicon concentrator (Lexington, MA, USA). NaN_3, Brij and $CaCl_2$ were added in order to achieve a final concentration of 0.1%, 0.05% or 5 mM. The samples were mixed with non-reducing buffer and loaded onto a 10% polyacryl amide/SDS gel containing 1 mg/ml gelatine (porcine skin type I, bloom 300, SIGMA G2500) and 107 ng/ml active MMP-2 protein

(Oncogene, Cambridge, MA, USA). After electrophoresis, the SDS was removed from the gel by incubation for 2 h in 2.5 % Triton X 100. The gel was incubated over night in 50 mM Tris HCl, pH 8.0, 50 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, and 0.02% NaN_3 at 37°C. The gel was stained with 0.5% Coomassie Brilliantblau (SIGMA R250, Deisenhofen, Germany), and bands of gelatinase-inhibitory activity representing TIMP-2 then appeared dark against the digested background. Reverse zymography only showed gelatinase-inhibitory activity in the Ad-TIMP-2 supernatants of infected cells and not in supernatants of non-infected or control virus infected cells.

c) The production of MMP-2 by tumour cells was proven by gelatine zymography. The CCS of various cell lines was collected and conditioned as described under (b). The zymography was done as described under (b), only MMP-2 was not added. Bands of the lyses showing gelatinolytic activity were visible against the dark background. Amongst several cell lines, LS 174 colon tumour cells manifested the strongest MMP-2 activity.

d) As proof of recombinant human TIMP-2 in the blood of nude mice, various doses of Ad-TIMP-2 were injected intravenously into the tail vein of nude mice. Blood samples were taken at various times and serum obtained. The samples were checked for TIMP-2 content by means of a TIMP-2 ELISAs (RPN 2618, Amersham Buchler, Braunschweig, Germany). The results show a dependence of the TIMP-2 expression on the applied amount of virus with a TIMP-2 concentration of 45 µg/ml and a dose of 3×10^{10} pfus (plaque forming units). The serum concentration of TIMP-2 was stable for 2 weeks. These results show that TIMP-2 is secreted after intravenous injection into the blood.

e) In order to check the extent of the gene transfer into the liver, adenoviruses were injected into the tail veins of nude mice. After three days, the animals were killed and the livers frozen in liquid nitrogen. For immuno-histochemical detection of TIMP-2, a monoclonal mouse antibody against human TIMP-2 (1:10, T2-101, Ab-1, Dianova, Hamburg, Germany) was used. An FITC-conjugated sheep anti-mouse antibody was used as a secondary antibody. The result of the staining showed an expression of -2

by 40% of the hepatocytes with a dose of 3×10^{10} pfu and of >80% with a dose of 6×10^{10} pfu. A similar gene transfer efficiency was documented after application of Ad-ßgal and subsequent X-gal staining.

f) The effectivity of intravenous application of Ad-TIMP-2 against LS 174 derived hepatic metastases was tested with the following experiments. Adenoviruses were applied 1 day before or 10 days after the induction of metastases. The induction of metastases was done by application of 2×10^{6} LS174 cells into the spleen of the animals. 5 weeks after induction of the metastases, the animals were killed and the tumour weights determined. In the preventive test approach, Ad-TIMP-2 or Ad-ßgal control virus in a dose of 3×10^{10} pfu, leading to about 40% liver cell infection, was administered on day 0 via the tail vein. The metastases were induced after 2 days via the above mentioned spleen injection of tumour cells. The experiment was ended after 4 weeks and the volumes of the liver tumours determined. It was seen that both in the untreated control group as well as in the group treated with the control virus, the majority of the animals manifested a metastasisation almost completely consuming the liver, whereas the majority of the animals treated with Ad-TIMP-2 were macroscopically free of tumours (Figure 1: untreated, Ad-ßgal treated (middle) and Ad-TIMP-2 treated animals (right)).

The quantitative evaluation resulted in a ratio of the mean figure of the tumour weights in the three test groups of 1:20 (Ad-TIMP-2: Ad-ßgal or untreated, Figure 2, dots correspond to individual animals, bars to the mean figures). The histopathological examination only showed individual micro-metastases in the macroscopically tumour-free animals of the Ad-TIMP-2 group. In this way, it can be seen that the gene-therapeutically mediated secretion of TIMP-2 by hepatocytes highly significantly limits both the quantity as well as the size of colorectal metastases in the nude mouse model. This finding was surprisingly unambiguous and speaks in favour of a central, irreplaceable function of MMP-2. As only about 40% of the hepatocytes were transduced in the approach described, it is obvious that a highly effective therapy exists here, presumably caused by local high concentrations of the active agent with an anti-metastatic effect on a number of levels (extravasion, infiltration and angiogenesis).

In a second experiment, the application of the therapeutic vectors was one week after the induction of metastases. In this case, the therapeutic efficiency was lower than with preventive administration of the vectors. The differences between the treatment group (TIMP-2) and control groups (Ad-ßgal or untreated) were however highly significant.

g) The livers of the animals in both experimental approaches were processed histologically in order to examine them for the standard tumour parameters apoptosis and proliferation and for the extent of angiogenesis.

To determine the angiogenesis, proliferation and apoptosis, paraffin slices of the liver were produced and stained with the following antibodies: to prove angiogenesis, a CD31 antibody (Daco, Hamburg, Germany) was used, to determine the percentage of proliferating cells an MIB-1 (Ki-67) antibody (dia 505, Dianova). The proof was done by means of a biotinylated second antibody and a horseradish conjugated avidine (Dako). To prove apoptosis, an ApopTag fluorescein in situ apoptosis kit (Intergen, Oxford, UK, TUNEL method) was used. De-paraffinised slices were pre-treated with proteinase K. Terminal deoxynucleotidyl transferase (TdT) was used for primary staining and anti-digoxigenin, conjugated on fluorescein, was used as a reporter molecule. Propidium iodide was used for counter-staining. Mitoses were counted on hematoxylin/hosin stained slices. 10 microscopic fields per animal were counted at an enlargement of x400 (MIB, mitoses, CD31) or with an oil immersion objective (x1000, TUNEL) using a Zeiss (Axioskop) fluorescence microscope (Carl Zeiss, Jena, Germany) and the mean values calculated. The mean values of all the data of animals with tumours in the individual treatment groups were calculated and Student's T tests were used for statistical analysis.

A significantly to highly significantly reduced proliferation rate and number of blood vessels as well as increased apoptosis rate were seen in Ad-TIMP-2 treated animals compared with untreated or control virus treated animals.

Further embodiments of the invention are concerned with vectors, promoters/enhancers, transgenes, trans-membrane anchors and target organs.

Vectors

Long-term expression of the transferred genes is above all essential if occult metastases exist, the reactivation of which can only occur after years of latency and with pre-operative metastasis prevention. If first-generation adenoviruses are used, the therapeutic effects are limited in time to a few weeks. Immunological defence reactions of the recipient are made responsible for this. These appear to be caused by the residual expression of adenoviral genes (Yang, Y. et al. (1994) Cellular immunity to viral antigens limits E1-deleted adenovirus for gene therapy. Proc. Natl. Acad. Sci. USA 91, 4407-4411.). A highly promising approach for the reduction of the immunogeneity is removal of all the coding adenoviral genome sections from the therapeutic vector (Chen, H.H. et al. (1997) Persistence in muscle of an adenoviral vector that lacks all viral genes, Proc. Natl. Acad. Sci. USA 94, 1645-1650). At the same time, the transport capacity of such viruses is considerably increased, with the result that a number of transgenes have room on one vector, making an attack on various positions of the metastasisation cascade possible. Such so-called helper dependent (HD), gutless, or minimal adenoviruses bearing TIMP-2 can presumably provide a long-term protection against organ metastasasation.

Further vectors permitting a longer-term external gene expression are retro-viruses and adeno-associated viruses (AAVs). Both viruses are not immunogenic and integrate necessarily (retro-viruses) or potentially (AAVs) into the genome of the host cell. Whereas the necessity of replication of the target cells and the difficulty of the generation of high-titre virus suspensions still cause problems in retroviruses, modern AAV vectors can already be used in the medium-term for gene transfer of protease inhibitors. Further promising vectors are lentivirus hybrid constructs as well as herpes simplex viruses, which have a high affinity to neuronal tissue and are therefore particularly suited to the treatment of brain metastases and glioblastoma.

Liposomes are to be emphasised among the non-viral vector systems.

A preferred embodiment of the invention is the modifications in the surface structure of viruses, enabling a re-targeting of the vectors. This is achieved by a suitable ligand being expressed on viral spikes, thus enabling a purposeful transduction of certain normal tissue. For example, cells expressing heparan can be purposefully transduced adenovirally by incorporation of a heparin domain.

8

Enhancers/Promoters

Enhancers/promoters active in the normal tissue to be protected can be used. In most cases, this involves the organ parenchyma. In individual cases, an expression of anti-tumour transgenes by under-represented cells can be sensible, i.e. the secretion of collagen, for example by fibroblasts, mentioned below.

A further possibility entails using promoters which are only activated following the addition of a substance foreign to the body. With such promoters, e.g. tetracyclin-dependent promoter elements or steroid-responsive elements, one has the possibility of only sporadically impregnating or selecting the most dangerous points in time for a metastasasation.

Transgenes

1. TIMP-2 is the suitable protein for the treatment of LS174 cell-derived metastases. MMP-2, which is inhibited by TIMP-2, is one of the most relevant proteases for tumour cell invasion. However, other cell lines also produce other MMPs, and this is also reflected in the protease pattern of human tumours. The extra-cellular matrix of the target organs is also structured differently, which makes varying demands on the tumour cell proteases. A generally valid approach must therefore also include other protease inhibition agents than TIMP-2, e.g. TIMP-1, PAI-1 or PAI-2. Modifications in structure of the naturally occurring inhibitors lead to increases of the effectivity or reductions of any side-effects. Such modifications comprise curtailments of the molecule or alterations of the sequence by replacement of individual bases of the DNA. For example, removal of a terminal (C-terminal) part of the TIMP-2 molecule also means removal of its undesired protease-activating function.

2. An alternative to inhibition of the degradation of the extra-cellular matrix (ECM) is reinforcement or modification thereof. Here, naturally occurring components of the extra-cellular matrix can be over-expressed. This includes the genes for the various collagens, fibronectin, laminin and genes, the products of which are responsible for the synthesis of non-protein components of the ECM. Further, components of the

9

ECM which are not normally expressed in the organ in question can be expressed at a different place and thus the organ specificity of metastases amended. Further, non-decomposable or difficult to decompose substances which mean an insurmountable obstacle for metastatic cells can be expressed.

Embodiment 2:

It has been known for a long time that metastases occur less frequently in cirrhotic livers than in normal livers. The cause of this is presumably the inhibition of the propagation of metastatic cells in fibrotic tissue. This connection can be made use of therapeutically and extended by gene transfer causing hepatic normal tissue to alter its micro-anatomy in such a way that a form of limited artificial cirrhosis/fibrosis is generated and thus metastatic cells are prevented from expanding. Collagen IV is the main component part of basal membranes. Nothing like this exists between hepatocytes and sinusoids. There is only little collagenic tissue in the space of Disse. A slight increase of the collagen content will distinctly limit the metastasisation capacity.

Vector construction: The two polypeptide chains generating the triple helix of the collagen fibrils are cloned into a minimal adenovirus shuttle vector. As a promoter, a tet-activator-responsive and doxycyclin-dependent promoter is used in order to keep the extent of transgene expression controllable. In addition, the tet-activator is also applied to the shuttle plasmide. A minimal adenovirus is produced using an arbitrary packaging system (=HDAD-tetColl)

Vector testing: Hepatic metastases are induced in nude mice by injection of LS 174 cells. The administration of HDAd-tetColl is done analogous to the methods in embodiment 1. The same applies for the evaluation.

3. Another way of blocking tumour cell invasion and motility is reinforcing the cell-cell and cell-matrix adhesions. The tight junctions with the proteins claudin and occludin, the desmosomes and adherence junctions with the main protein cadherin, integrins, which react above all with components of the ECM, the immunoglobulin superfamily, the selectins and the muzins are to be mentioned.

Embodiment 3:

10

It has already been shown that E-cadherin is responsible for the interaction of epithelial cells and a loss of E-Cadherin by cells in the primary tumour supports metastasisation (Birchmeier, W. (1995) E-cadherin as a tumour (invasion) suppressor gene. Bioessays 17, 97-99). An expression of E-cadherin by normal cells leads, on the one hand, to an adhesion with the tumour cells and to an inhibition of their motility and further to a reinforcement of the adhesion within the normal cells.

Vector construction: A first-generation adenovirus which carries the E-cadherin gene under the control of the RSV promoter is constructed: Ad-RSV-E-Cad.

In vitro testing: For function testing, A2 cells are transduced with Ad-RSV-E-Cad. An increase of the adhesion is determined by establishing the period of time which trypsin needs to separate the cells.

Vector testing: Hepatic metastases are induced in nude mice by injection of LS 174 cells. The administration of HDAd-tetColl is done analogous to the methods in embodiment 1. The same applies for the evaluation.

Transgenes with membrane anchor sequence

Suicide genes are used within the sense of the invention for impregnation of normal tissue. For this, they must be equipped with a membrane anchor sequence in order to be effective extra-cellularly and also to be able to toxify an applied pro-drug extra-cellularly.

Embodiment 4:

The gene for the suicide gene cytosin desaminase under the control of the HNFAlbumin promoter is provided with a membrane anchor sequence, with the result that it is expressed on the membrane following transfection. This expression cassette is cloned into an AAV-shuttle plasmide and an AAV produced making use of an arbitrary helper system (=AAV-HNFAlb-CD-Tm).

In vitro testing: A2 cells are transduced with AAV-HNFAlb-CD-Tm. 24 h after transduction, pro-drug 5-FC is added to the cell culture supernatant (CCS). 5-FC is now transferred into the cytotoxic 5-FU by the CD on the membrane. The supernatant is collected after 24 h and added to the LS174 cell line as well as

11

quiescent primary hepatocytes. After a further 72h, cell counts are made and the extent of the apoptosis determined.

In vivo testing: vector application and determination of the therapy efficiency are done analogous to embodiment 1.

Target organs

On the basis of the outstanding infectibility of the liver in systemic administration of adenoviruses and the high clinical relevance of the treatment of colorectal metastases, the method according to the invention was developed on the basis of the disease model described above. The invention can also be applied for other tumour diseases. The most frequent clinical pictures include liver, lung, bone and brain metastases with mamma carcinomas with latency periods of up to 10 years after removal of the primary tumour, a period which has expired unused up to now, brain metastases with bronchial carcinomas and bone metastases with prostate carcinomas.

Further, there are primary tumours which are primarily inoperable due to their advanced stage, such as frequently glioblastoma and hepatocellular carcinomas. Protection of the surrounding normal tissue according to the above mentioned principle is imaginable here as a life-extending measure.

Legend for the figures:

Figure 1: Prevention of liver metastasasation by systemic application of Ad-TIMP-2. On day 0, 3×10^{10} pfu Ad-TIMP-2 or Ad-ßgal are applied into the tail vein of nude mice. 3 days later, the animals are given an intra-splenal injection of LS174 colon carcinoma cells to induce liver metastases. Representative in situ photographs of untreated (left), Ad-ßgal treated (middle) and Ad-TIMP-2 treated (right) animals after 5 weeks.

Figure 2: Method as in Figure 1. After 5 weeks, the animals are killed and the tumour masses determined. Dots are individual animals, bars are the mean values.